DEVELOPMENTAL DYNAMICS 235:1884–1894, 2006

RESEARCH ARTICLE

Establishment of the Epaxial–Hypaxial Boundary in the Avian Myotome

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Trunk skeletal muscles are segregated into dorsomedial epaxial and ventrolateral hypaxial muscles, separated by a myoseptum. In amniotes, they are generated from a transient structure, the dermomyotome, which lays down muscle, namely the myotome underneath. However, the dermomyotome and myotome are dorsoventrally continuous, with no morphologically defined epaxial–hypaxial boundary. The transcription factors En1 and Sim1 have been shown to molecularly subdivide the amniote dermomyotome, with En1 labeling the epaxial dermomyotome and Sim1 the hypaxial counterpart. Here, we demonstrate that *En1* and *Sim1* expression persists in cells leaving the dermomyotome, superimposing the expression boundary onto muscle and skin. *En1*-expressing cells colonize the myotome initially from the rostral and caudal lips, and slightly later, directly from the de-epithelializing dermomyotomal center. *En1* expression in the myotome is concomitant with the appearance of *Fgfr4*/*Pax7*-expressing mitotically active myoblasts. This finding suggests that *Fgfr4*$^+$/*Pax7*$^+$/*En1*$^+$ cells carry their expression with them when entering the myotome. Furthermore, it suggests that the epaxial–hypaxial boundary of the myotome is established through the late arising, mitotically active myoblasts. *Developmental Dynamics 235:1884–1894, 2006.* © 2006 Wiley-Liss, Inc.

Key words: *En1*; *Sim1*; *Paraxis*; *Pax7*; *Pax3*; *Alx4*; *Myf5*; *Fgfr4*; somite; dermomyotome; myotome; dermatome; skeletal muscle; epaxial; hypaxial; boundary; fate-map; chick embryo

Accepted 24 March 2006

INTRODUCTION

In higher vertebrates, trunk skeletal muscles are segregated into separately innervated epaxial (deep muscles of the back) and hypaxial muscles (intercostal, body wall, and limb muscles), both physically separated by a myoseptum, termed the thoracolumbar fascia in humans, septum laterale in amniotes, and horizontal myoseptum in fishes (Goodrich, 1958; Young, 1962; Christ and Ordahl, 1995; Gray, 1995; Devoto et al., 1996; Blagden et al., 1997; Gossler and Hrabé de Angelis, 1998; Stickney et al., 2000; Goulding et al., 2002; Shirasaki and Pfaff, 2002). Epaxially–hypaxially segregated muscle is a prerequisite for complex three-dimensional movement, and has been established during the agnathan–gnathostome transition (Goodrich, 1958; Young, 1962).

In all vertebrates, trunk skeletal muscles develop from the segmented paraxial mesoderm termed somites. In jaw-bearing fishes, the somite readily differentiates into epaxially–hypaxially segregated muscle (Devoto et al., 1996; Blagden et al., 1997; Stickney et al., 2000). However, this does not occur in amniotes. Here, the somite differentiates along the dorsal–ventral axis into a dorsally located dermomyotome, the source of dorsal dermis and muscle, and the ventrally located sclerotome, the source of the vertebral column and ribs (Christ and Ordahl, 1995; Gossler and Hrabé de Angelis, 1998; Pourquié, 2001). The dermomyotome forms the first embryonic muscle, the myotome, by depositing myoblasts underneath in waves (Christ et al., 1983; Denetclaw et al., 1997; Kahane et al., 1998a; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Huang and Christ, 2000;

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Grant sponsor: Human Frontier Science Program; Grant number: R6Y0056/2004-C201; Grant sponsor: European Network of Excellence Myores; Grant Number: EU LSHG-CT-2004-511978 MYORES; Grant sponsor: Association Française contre les Myopathies; Grant number: CL/NM/2005.2088/ Following number 11378, Groupe A07/06/05 and CA 01/07/05, DRM05/BUIOCE/REFAP/MUSAI/ANGLE.
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DOI 10.1002/dvdy.20832
Published online 5 May 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Function of Marker Genes

Gene	Function
En1	Lead marker for epaxial cells
Sim1	Lead marker for hypaxial cells
Paraxis	Required for epithelialization of early somites and specification of myogenic precursors in the dermomyotome
Pax7	Commits pluripotent stem cells to the myogenic lineage and specifies satellite cells
Pax3	Master regulator of trunk myogenesis and is required for the delamination of migratory muscle precursors at limb levels
Alx4	Required for the anteroposterior patterning of the limbs and is associated with dermal fate
Myf5	Required for the determination of myoblasts
Fgfr4	Lead marker for mitotically active muscle progenitors

Cinnamon et al., 2001; Gros et al., 2004). The first wave contains postmitotic cells and arises from the dorsomedial wall of the epithelial somite (Kahane et al., 1998b; Cinnamon et al., 1999). According to Kalcheim and colleagues, these cells spread underneath the dorsomedial–ventrolateral extent of the dermomyotome (Kahane et al., 1998b; Cinnamon et al., 1999), and act as a scaffold for the intercalation and alignment of a second wave of postmitotic cells that arises from all four edges of the dermomyotome (Kahane et al., 1998a; Cinnamon et al., 1999; Gros et al., 2004). In this second wave, the cells remain true to origin in that the dorsomedial lip contributes to the dorsomedial, the ventrolateral lip to the ventrolateral aspect of the myotome, and cells from the rostrocaudal lips project into the myotome directly underneath (Denetclaw et al., 1997; Kahane et al., 1998a; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Gros et al., 2004; Huang and Christ, 2000). The first two waves create a primary, postmitotic myotome, which is then populated by a third wave of progenitor cells that enter from the rostral and caudal lips of the dermomyotome, are mitotically active, and express *Fgfr4* (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001). Finally, when the center of the dermomyotome de-epithelializes, the myotome experiences a massive influx of a fourth wave of mitotically active, *Fgfr4*-expressing muscle precursors (Ben-Yair and Kalcheim, 2005; Gros et al., 2005). Due to their mitotic activity, these cells will eventually constitute the bulk of the muscle (Ben-Yair and Kalcheim, 2005; Gros et al., 2005).

The dermomyotome as well as the myotome are dorsoventrally continuous structures with no morphologically defined epaxial–hypaxial boundary (Christ and Ordahl, 1995; Gossler and Hrabé de Angelis, 1998). However, labeling experiments indicated that epaxial–hypaxial cells arise from distinct cell lineages during gastrulation (Selleck and Stern, 1991; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002). Moreover, evidence from cell lineage restriction and cell sorting assays indicated that the mature dermomyotome and myotome comprises distinct epaxial and hypaxial compartments (Selleck and Stern, 1991; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002; Cheng et al., 2004). Thus, an epaxial–hypaxial subdivision, although morphologically concealed, is present in the amniote somite. Nevertheless, the initially laid down myotomal scaffold (first wave), at least at flank levels, seems to originate from the medial/epaxial side of the somite only (Kahane et al., 1998b; Cinnamon et al., 1999). Moreover, the fate of prospective medial/epaxial and lateral/hypaxial is not determined in the early somite and can be changed when the signals patterning the somite mediolaterally are altered (Pourquié et al., 1996; Dietrich et al., 1998; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002). This finding indicates that the epaxial–hypaxial somite boundary is established over time.

Recent studies demonstrated that the epaxial–hypaxial boundary of the dermomyotome is molecularly defined by the differential expression of the homeobox containing transcription factor En1 and the basic helix–loop–helix (bHLH) transcription factor Sim1, with En1 demarcating the epaxial and Sim1 the hypaxial domains (Cheng et al., 2004). Triggered by the lateral mesoderm, *Sim1* is expressed in the entire lateral somite half from early stages onward (Pourquié et al., 1996). *En1* expression, initiated by a complex regulatory network involving the notochord, neural tube, and surface ectoderm, appears in the dermomyotome at somite stages VIII–X (Olivera-Martinez et al., 2002; Cheng et al., 2004). At this stage, the *En1*- and *Sim1*-expressing parts of the dermomyotome form a compartment boundary (Cheng et al., 2004). This finding suggests that *En1* is the key marker for the beginning of epaxial–hypaxial segregation of the somite. Eventually, *En1* expression also appears in the myotome, with the expression boundary aligned with the expression boundary in the overlying dermomyotome (Cheng et al., 2004). DiI-DiO labeling and quail–chick transplantation experiments indicated that all *En1*-expressing cells arise from the epaxial part of the somite (Cheng et al., 2004). However, how *En1*-expressing cells emerge in the myotome is not understood.

En1 expression commences in the dermomyotome just before mitotically active muscle precursors begin to immigrate into the myotome (Olivera-Martinez et al., 2002; Cheng et al., 2004). We, therefore, hypothesized that these cells may carry the dermomyotomal *En1* expression with them, thereby establishing the molecularly defined epaxial–hypaxial boundary in the myotome. To test this possibility, we precisely mapped the temporospatial appearance of *En1* expression in the myotome, compared its expression with the expression patterns of markers for the dermomyotome and myotome, specifically that of *Fgfr4*, the marker for late emerging mitotically active myo-

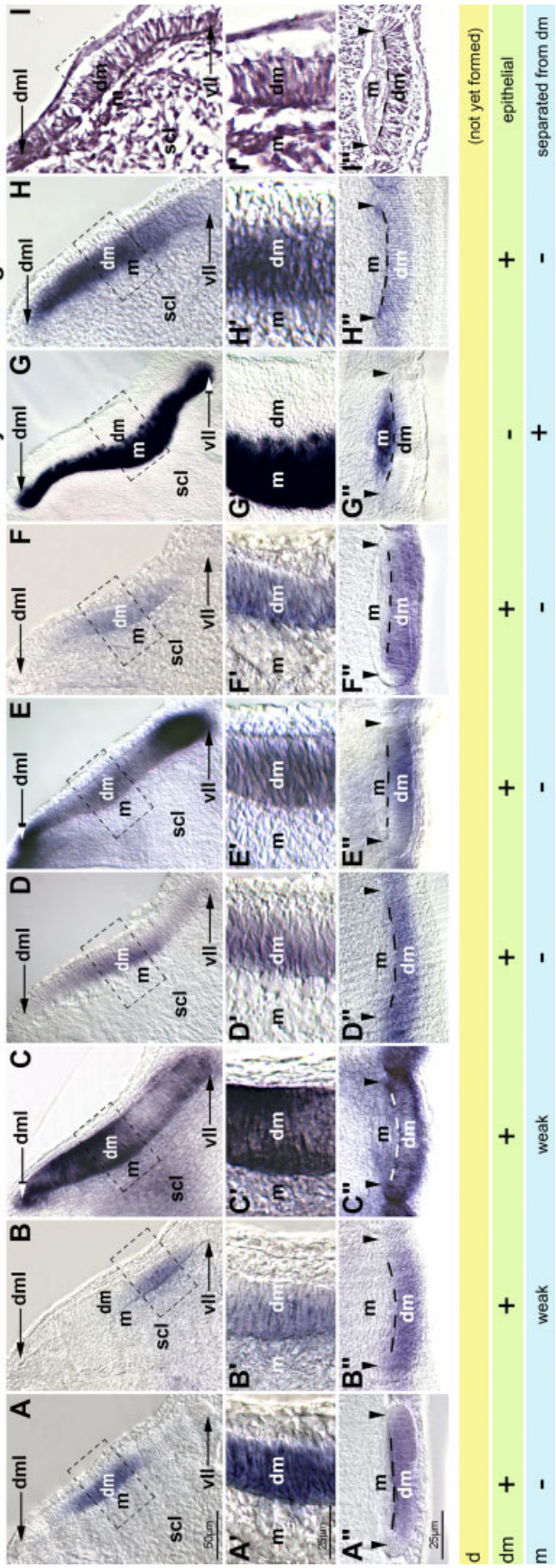
En1
Sim1
Paraxis
Pax7
Pax3
Alx4
Myf5
Fgfr4
H&E
dml
scl
vll
dm
m
d
50μm
25μm
(not yet formed)
epithelial
separated from dm
weak

Fig. 1.

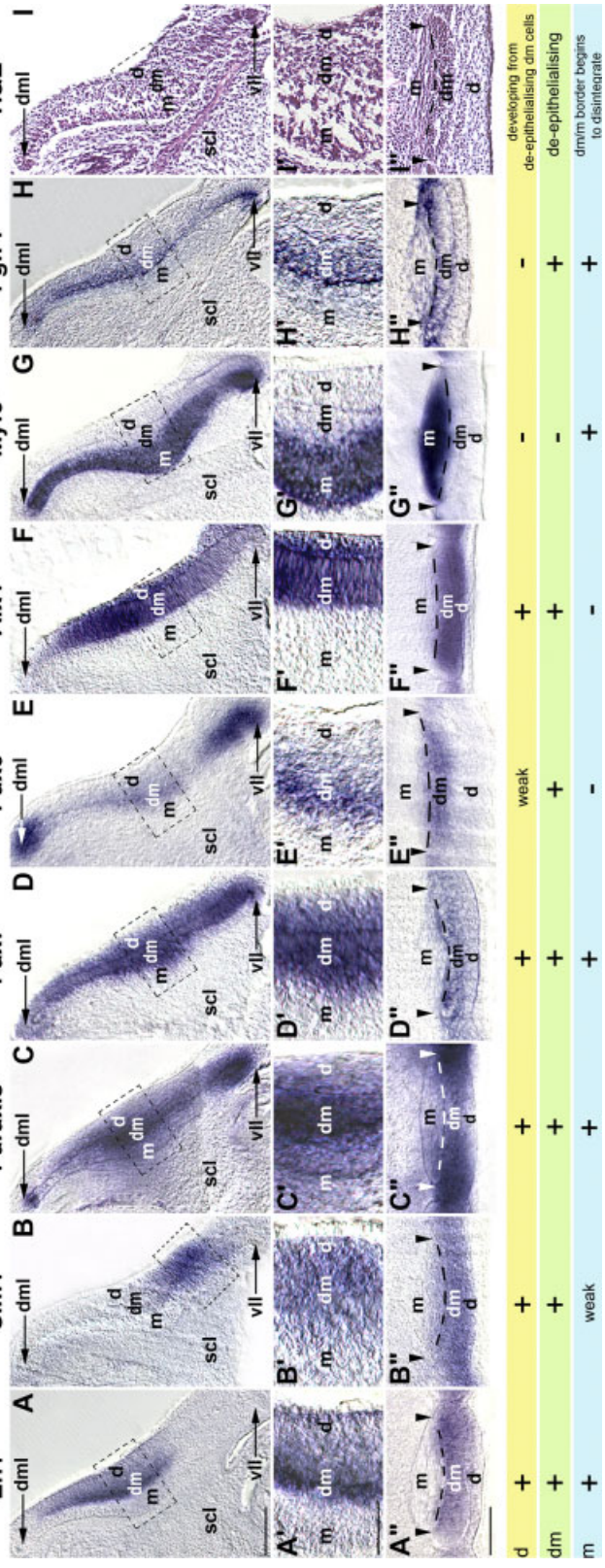
En1
Sim1
Paraxis
Pax7
Pax3
Alx4
Myf5
Fgfr4
H&E
dml
scl
vll
dm
m
d
developing from de-epithelialising dm cells
de-epithelialising
dm/m border begins to disintegrate
weak

Fig. 2.

blasts (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001). We also fate-mapped cells in different regions of the *En1*-expressing dermomyotome and investigated the histological changes within the dermomyotome and myotome. We show that *En1*-expressing cells enter the myotome first from the rostral and caudal lips of the dermomyotome. Once the dermomyotome de-epithelializes to form the dermis, *En1*-expressing cells also directly enter the myotome beneath. *En1* expression in the myotome is concomitant with the appearance of *Fgfr4*-expressing myoblasts that belong to the third and fourth wave category of mitotically active muscle precursors (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001; Ben-Yair and Kalcheim, 2005; Gros et al., 2005). This finding suggests that the epaxial–hypaxial boundary of the dermomyotome is superimposed onto the myotome when the mitotically active *Fgfr4*/*En1*-positive myoblasts (which in addition express *Paraxis*/*Pax7*) populate the initially epaxially–hypaxially indistinct primary myotome.

Fig. 1. Marker gene expression and somite morphology at embryonic day 3/Hamburger and Hamilton stage 20 (E3/HH20). **A–H:** Fifty-micrometer Vibratome cross-sections of E3/HH20 flank somites, stained for the expression of *En1*, *Sim1*, *Paraxis*, *Pax7*, *Pax3*, *Alx4*, *Myf5*, and *Fgfr4* as indicated on the top of the panel; the dorsomedial lip of the dermomyotome is to the top left, the ventrolateral lip to the bottom right corner. **I:** Hematoxylin-eosin–stained 12-μm paraffin cross-section orientated as in A–H. The dashed boxes in A–I correspond to the amplified area shown in A′–I′. A″–I″: Vibratome (A″–H″) and paraffin (I″) frontal sections of E3/HH20 flank somites; medial is to the top, lateral to the bottom, arrowheads indicate rostral and caudal edges, broken lines indicate the dm/m border. In the bars at the bottom of the figure, the presence (+) or absence (−) of marker gene expression and the morphology of dermomyotome, myotome, and dermatome is summarized. Note that at HH20, expression of *En1*, *Pax7*, *Pax3*, *Alx4*, and *Fgfr4* is restricted to the dermomyotome, *Sim1* and *Paraxis* show low-level expression also in the myotome and sclerotome, *Myf5* expression is confined to the myotome. Importantly, *En1* labels the epaxial, and *Sim1* labels the hypaxial side of the dermomyotome; their expression domains abut. The dermomyotome is epithelially organized and well-separated from the myotome. dm, dermomyotome; dml, dorsomedial dermomyotomal lip; H&E, haematoxylin–eosin staining; m, myotome; scl, sclerotome; vll, ventrolateral dermomyotomal lip. Scale bars = 50 μm in A (applies to A–I), 25 μm in A′,A″ (applies to A′–I′ and A″–I″, respectively).

Fig. 2. Marker gene expression and somite morphology at embryonic day 3.5/Hamburger and Hamilton stage 22 (E3.5/HH22). **A–I″:** Cross-sections (A–I), higher magnification of cross-sections(A′–I′), frontal sections (A″–I″) of E3.5/HH22 flank somites, orientation as in Figure 1. Note, the dermomyotome has begun to de-epithelialize; the mesenchymal dermis precursors accumulate underneath the surface ectoderm and constitute the dermatome. These cells maintain expression of *En1*, *Sim1*, *Paraxis*, *Pax7*, *Alx4*, but down-regulate *Pax3* and *Fgfr4*. The myotome is thicker than at E3 due to the continuous influx of muscle precursors. Note that cells entering the myotome from the rostrocaudal edges of the dermomyotome maintain expression of *En1*, *Pax7*, and *Fgfr4*, but they down-regulate *Alx4*. The cells express *Myf5* weakly; this marker predominantly labels differentiating cell in the center of the myotome. Significantly, the expression of *Fgfr4* continues in myogenic cells, that of *Alx4* continues in dermal cells. In contrast, *En1* and *Sim1* expression encompasses both lineages but retains the epaxial–hypaxial expression boundary. d, dermatome. Other abbreviations as in Figure 1. Scale bars = 50 μm in A (applies to A–I), 25 μm in A′,A″ (applies to A′–I′ and A″–I″, respectively).

RESULTS

En1 expression commences in the dermomyotome of somites stages VIII–X; the regulatory network controlling this expression has been fairly well characterized (Olivera-Martinez et al., 2002; Cheng et al., 2004). However, when and how *En1*-expressing cells appear in the underlying myotome, and whether myotomal *En1* expression is associated with any of the waves of muscle precursors entering the myotome, is unclear. We, therefore, (1) comparatively analyzed the expression of dermomyotomal and myotomal markers, (2) determined the histological appearance of the dermomyotome and myotome, and (3) traced *En1*-expressing cells with fluorescent dyes.

Comparative Expression Analysis of Epaxial–Hypaxial Markers in the Dermomyotome and Myotome and the Morphology of Somites

Embryos were analyzed from embryonic day 3/Hamburger and Hamilton stage 20 (E3/HH20), when *En1* is robustly expressed in the dermomyotome of stage XV–XVIII flank somites (Olivera-Martinez et al., 2002; Cheng et al., 2004), to E4.5/HH25, when the dermomyotome is fully de-epithelialized (Cheng et al., 2004; Ben-Yair and Kalcheim, 2005; Gros et al., 2005). A list of markers and their function is provided in Table 1.

Marker Gene Expression and Somite Morphology at E3/HH20

At E3, the expression of *En1* (Fig. 1A–A″) was restricted to the dermomyotome, bordering the expression domain of *Sim1* (Fig. 1B–B″). Thus, in line with previous studies (Cheng et al., 2004), the epaxial–hypaxial boundary is molecularly defined in the dermomyotome, but not yet in the myotome where *En1* was absent and *Sim1* was weakly expressed in the lateral domain. The bHLH transcription factor Paraxis, which is required to establish and maintain the epithelial characteristics of the somite and to specify myogenic precursors in the dermomyotome (Burgess et al., 1995; Šošic et al., 1997; Wilson-Rawls et al., 1999), was expressed throughout the dermomyotome, with up-regulated expression within the *En1* domain and in all four dermomyotomal lips (Fig. 1C–C″; weak expression is found in the myotome and sclerotome). The expression of *Pax7*, a paired and homeobox containing gene demarcating mitosis-competent myoblasts and muscle-derived stem cells known as satellite cells (Asakura et al., 2002; Oustanina et al., 2004; Ben-Yair and Kalcheim, 2005; Gros et al., 2005), was restricted to the dermomyotome, with strong expression in the central dermomyotome overlapping the expression domain of *En1* (Fig. 1D–D″). Its paralogue *Pax3*, a dermomyotomal marker and master regulator for trunk myogenesis (Goulding et al., 1994; Tajbakhsh et al., 1997; Relaix et al., 2004), was expressed in the whole dermomyotome with strongly up-regulated expression in the dorsomedial and ventrolateral lips and a more moderate up-regulation in the dermomyotomal center (Fig. 1E–E″). Expression of the homeobox containing gene *Alx4* was also confined to the dermomyotome (Takahashi et al., 1998; Cheng et al., 2004), encompassing the expression domains of *En1* and *Sim1* but not the *Pax3*-expressing dorsomedial and ventrolateral dermomyotomal lips (Fig. 1F–F″). The bHLH transcription factor Myf5, which belongs to the family of muscle deter-

mining factors and in chick is the first of them to be expressed in the myotome, labeled the differentiating muscle precursors entering from the dorsomedial and ventrolateral dermomyotomal lips plus the myotome proper (Fig. 1G–G″). In contrast, expression of the Fgf receptor 4 (*Fgfr4*; Fig. 1H–H″), the lead marker for mitotically active myoblasts (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001; Ben-Yair and Kalcheim, 2005; Gros et al., 2005), was still confined to the dermomyotome, encompassing the expression domains of both *En1* and *Sim1*. Histological analysis of the somites at this developmental stage revealed that the dermomyotome is epithelially organized and well-separated from the underlying, thin layer of myotomal cells (Fig. 1I–I″).

Marker Gene Expression and Somite Morphology at E3.5/HH22

At E3.5, *En1* expression had commenced in the myotome, labeling its rostrocaudal edges (Fig. 2A–A″). The dorsoventral extent of the myotomal expression matched that of the expression domain in the dermomyotome. In both tissues, *En1* expression abutted that of *Sim1* (Fig. 2B–B″), suggesting that a molecular epaxial–hypaxial boundary was now developing in the myotome. Concomitant with the appearance of *En1* expressing cells, expression of *Paraxis* (Fig. 2C–C″) and *Pax7* (Fig. 2D–D″) commenced at the rostrocaudal edges of the myotome. Moreover, this region now expressed *Fgfr4* (Fig. 2H–H″), indicating that mitotically active muscle precursors began to populate the myotome (Kahane et al., 2001). However, the expression of *Fgfr4* continued to encompass both the *En1* and *Sim1* expression domains, indicating that there is an *Fgfr4*/*En1*$^+$ and a *Fgfr4*/*Sim1*$^+$ cell population.

In contrast to *En1*, *Paraxis*, *Pax7*, and *Fgfr4*, expression of *Pax3* (Fig. 2E–E″) and *Alx4* (Fig. 2F–F″) remained in the dermomyotome, and expression of *Myf5* (Fig. 2G–G″) remained in the myotome. However, two changes were apparent in these domains: first, due to the continuous influx of myotomal cells, the myotome increased in thickness (compare Figs. 1, 2G–G″). Second, due to the beginning of the de-epithelialization process, the dermomyotome developed a two-layered appearance with more densely packed cells at the border of the myotome and more loosely packed, mesenchymal dermis precursors (which we refer to as dermatome) underneath the ectoderm. This observation was confirmed by the histological analysis (Fig. 2I–I″).

Marker Gene Expression and Somite Morphology at E4/HH24

At E4, the de-epithelialization of the dermomyotome was further advanced and a significant amount of mesenchymal dermis precursors had accumulated (Fig. 3I–I″). They continued to strongly express *Alx4* (Fig. 3F–F″), while *Paraxis* (Fig. 3C–C″), *Pax7* (Fig. 3D–D″), *Pax3* (Fig. 3E–E″), and *Fgfr4* (Fig. 3H–H″) expression declined. *En1* expression remained strong in the epaxial domain (Fig. 3A–A″), as did *Sim1* expression in the adjacent hypaxial domain (Fig. 3B–B″).

The dorsomedial and ventrolateral lips of the dermomyotome were still epithelial and expressed *Paraxis* (Fig. 3C–C″), *Pax7* (Fig. 3D–D″), *Pax3* (Fig. 3E–E″), and *Fgfr4* (Fig. 3H–H″). In the center of the somite, the remnant of the densely packed dermomyotomal cells was visible. However, the boundary between this domain and the underlying myotome had vanished (Fig. 3I′). Within the dermomyotome, cells expressed *En1* (Fig. 3A–A″; epaxial side), *Sim1* (Fig. 3B–B″; hypaxial side), *Paraxis* (Fig. 3C–C″), *Pax7* (Fig. 3D–D″), *Pax3* (Fig. 3E–E″), *Alx4* (Fig. 3E–E″), and *Fgfr4* (Fig. 3H–H″). With the exception of *Alx4*, the expression domains of these markers began to spread into the myotome underneath (Fig. 3A′,B′,C′,D′,E′,H′). On frontal sections that pass the center of the somite within the epaxial domain (Fig. 3A″,C–I″), it was evident that *En1*/*Paraxis*/*Pax7*/*Pax3*/*Fgfr4* expression demarcated the myotomal–dermomyotomal interface. Moreover, *En1*/*Paraxis*/*Pax7*/*Fgfr4* signals were spreading between the myofibers. Expression of these markers had also spread from the rostrocaudal edges, now covering the medial, sclerotome-facing surface of the myotome. Thus, mitotically active muscle precursors surrounded a core of *Myf5* expressing, differentiating cells, intermingling with them at the dorsal, dermomyotome-facing side (Fig. 3G–G″).

Sim1 expression followed the ventrolaterally outgrowing hypaxial myotome, overlapping with signals for *Pax3* (Fig. 3B,E). *Sim1* expression also spread laterally into the lateral mesoderm-derived dermis, here, however, confined to a thin layer of tissue directly beneath the surface ectoderm. Thus, *Sim1* remains associated with ventral/hypaxial structures.

Marker Gene Expression and Somite Morphology at E4.5/HH25

At E4.5, de-epithelialization of the dermomyotome was almost complete (Fig. 4I–I″). Significant changes in the expression of markers occurred in the differentiating dermis. Dermatomal *Alx4* expression was now continuous with *Alx4* signals in the lateral mesoderm, with stronger expression still in the dermatome (Fig. 4F–F″). *Sim1* expression was spreading medially/epaxially; however, the strongest *Sim1* expression was still at the border to the *En1* domain (Fig. 4A,B). Importantly, the lateral border of the *En1* expression domain was maintained, suggesting that the molecular subdivision at least of the myotome persisted.

The dorsomedial and ventrolateral lips of the dermomyotome were still epithelial and continued to express *Paraxis* (Fig. 4C), *Pax7* (Fig. 4D), *Pax3* (Fig. 4E), and *Fgfr4* (Fig. 4H). Within the dermomyotome proper, expression of *Fgfr4* began to decline (Fig. 4H–H″). In the myotome, however, *Fgfr4* signals encompassed the expression domains of both *En1* and *Sim1* as before (Fig. 4A,B,H). Nevertheless, the expression of *En1*/*Paraxis*/*Fgfr4* had almost completely disappeared from the rostrocaudal edges as cells derived from this region settle on the medial, sclerotome-facing surface of the myotome (Fig. 4A″,C″,H″).

The expression of *Pax3* had spread into the myotome from the dermomyotomal center (Fig. 4E–E″). *Pax7*

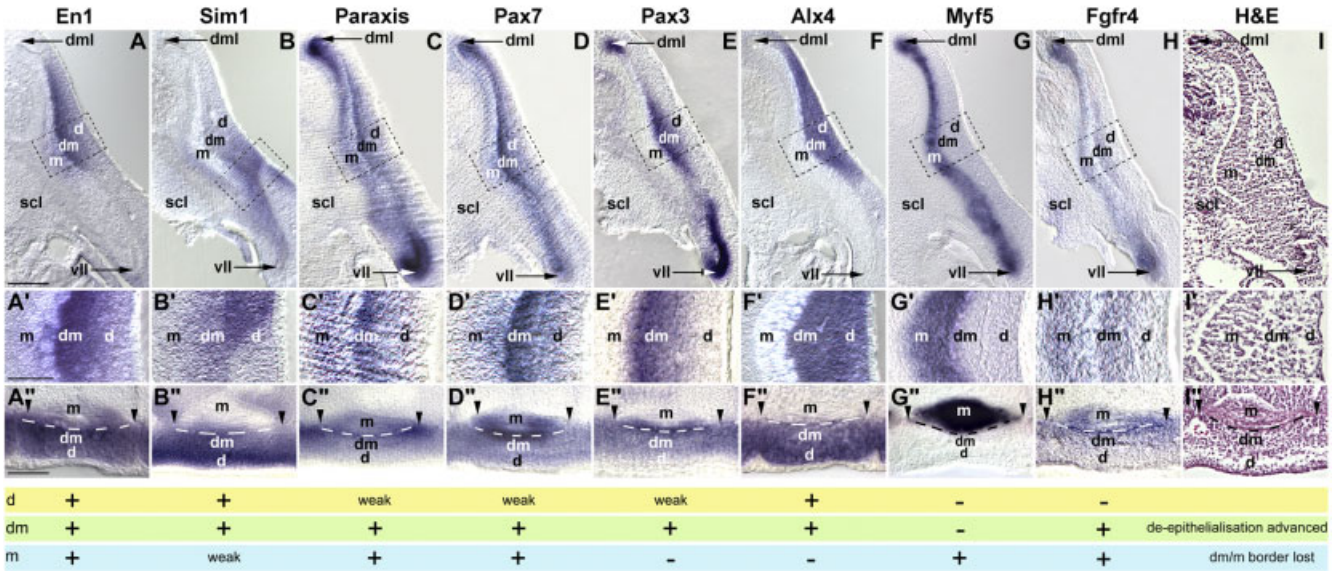

Fig. 3. Marker gene expression and somite morphology at embryonic day 4/Hamburger and Hamilton stage 24 (E4/HH24). **A–I″:** Cross-sections (A–I), higher magnification of cross-sections (A′–I′), and frontal sections (A″–I″) of E4/HH24 flank somites; orientation, scale bars, and abbreviations as in Figures 1 and 2. The de-epithelialization of the dermomyotome is further advanced and the border between dermomyotome and myotome disappeared. Notably, expression of *En1*, *Sim1*, *Paraxis*, *Pax7*, and *Fgfr4* is found not only in muscle precursors at the rostrocaudal edges of the somite but also spreading directly from the dissolving dermomyotome into the myotome. *Alx4* expression is confined to dermomyotomal and dermal cells. Note that the expression boundary of *En1* (A) and *Sim1* (B) is maintained as cells from the de-epthelializing central dermomyotome contribute to myotome and dermatome.

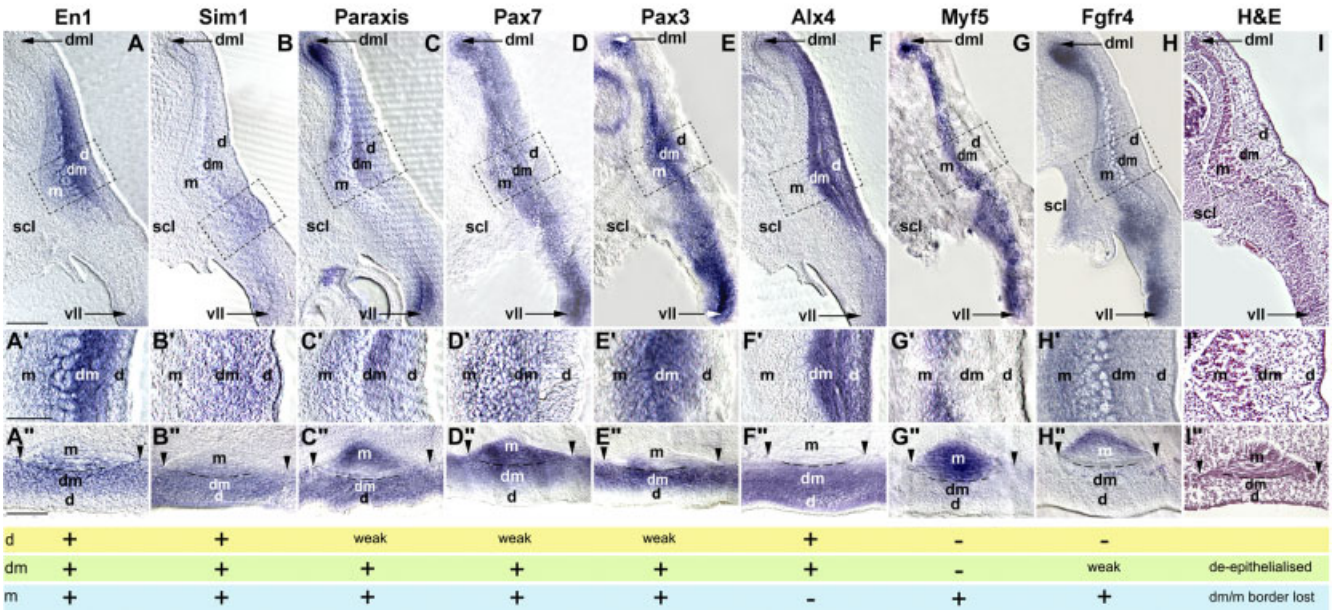

Fig. 4. Marker gene expression and somite morphology at embryonic day 4.5/Hamburger and Hamilton stage 25 (E4.5/HH25). **A–I″:** Cross-sections (A–I), higher magnification of cross-sections (A′–I′), and frontal sections (A″–I″) of E4.5/HH25 flank somites; orientation, scale bars, and abbreviations as in Figures 1 and 2. The de-epithelialization of the dermomyotome is almost complete; only the dml and vll are still epithelial and continue to express *Paraxis*, *Pax7*, *Pax3*, and *Fgfr4*. Note the significant spread of *En1*, *Sim1*, *Paraxis*, *Pax7*, *Pax3*, and *Fgfr4* staining from the central dermomyotome into the myotome, only partially overlapping, however, with the staining of *Myf5*. Also note that expression of *Paraxis*, *Pax3*, and *Pax7* is being shed from the dermatome, which continues to express *Alx4*. In the dermomyotome and its derivatives, the expression boundary of *En1* and *Sim1* is maintained.

expression, and for the epaxial domain, *En1* expression, was maintained in myoblasts interspersed between the myofibers. Thus, by E4.5, there was significant penetration of the myotome by cells from the central dermomyotomal sheet, which first express *Paraxis*, *Pax7*, *Pax3*, and *Fgfr4*, then predominantly *Pax7*. The epaxial component of these cells is characterized by prolonged *En1* expression.

Fate-Mapping *En1*-Expressing Cells in Different Regions of the Dermomyotome

Our study so far showed that *En1* expression commences in the myotome, concomitant with the emergence of *Fgfr4*/*Paraxis*/*Pax7* expression at the rostrocaudal borders. Subsequently, *Fgfr4*/*Paraxis*/*Pax7*/*Pax3*/*En1*-expressing cells seem to enter the myotome directly from the de-epithelializing dermomyotome. These cells maintain *Pax7*/*En1* expression for a prolonged period. To confirm the route of entry of *En1*-expressing cells into the myotome, we traced these cells using fluorescent labeling (Fig. 5A,B,E,F,I,J). The rostral (or caudal) border of the area known to express *En1* was labeled with DiO, the dermomyotomal center with DiI, as indicated in Figure 5A. The dyes were administered to flank somites VIII–XII of HH17^{+}–18^{-} chick embryos as shown in Figure 5B,F,J. The embryos were incubated to E3.5 and E4 and analyzed for the distribution of the fluorescent cells.

At E3.5, cells labeled at the rostral (Fig. 5C) or caudal (not shown) edge of the dermomyotome had contributed to the myotome; some had differentiated into elongated myocytes orientated parallel to the rostrocaudal axis of the somite (open white arrowheads in Fig. 5C). Cells labeled in the dermomyotomal center, by contrast, remained closely packed together (Fig. 5C). Occasionally, cells from this region were found at the myotomal–dermomyotomal interface (arrow in Fig. 5G) as the central dermomyotome began the process of de-epithelialization, however, most remained in the dermomyotome (Fig. 5K).

At E4 (i.e., 36 hr after the initial labeling), a larger proportion of cells from the rostrocaudal edges of the *En1* domain had differentiated into elongated myocytes (open white arrowheads in Fig. 5D); the remaining cells had spread over the medial, sclerotome-facing surface of the myotome. Cells from the central dermomyotome had spread dorsally, contributing to the mesenchymal dermatome. Importantly, cells from the central territory had also contributed to the myotome. As seen in both cross- and frontal sections, these cells pass through the myotome–dermomyotome interface as they move ventrally into the myotome (Fig. 5H,L, arrows). They largely remained mesenchymal, although occasionally, differentiation into elongated myocytes was observed (Fig. 5D, yellow open arrowhead).

In summary, the colonization of the myotome by *En1*-expressing dermomyotomal cells occurs by means of two routes. First, *En1*-expressing cells appear in the myotome at E3.5 from the rostral and caudal edges. Second, *En1*-expressing cells appear in the myotome at E4 from the dissociating central dermomyotome.

DISCUSSION

Complex, three-dimensional movement relies on separately innervated and physically segregated epaxial and hypaxial muscles (Goodrich, 1958). In amniotes, epaxial–hypaxial muscles develop from a morphologically continuous embryonic muscle, the myotome, which in turn is generated by the morphologically continuous dermomyotome (Christ and Ordahl, 1995; Gossler and Hrabé de Angelis, 1998). However, evidence is accumulating that the transcription factors En1 and Sim1 may be involved in the molecular epaxial–hypaxial subdivision of the dermomyotome and myotome as prelude to the subdivision of muscle (Cheng et al., 2004). In the avian dermomyotome, expression of *En1* and the lead hypaxial marker *Sim1* abut each other, with *En1* demarcating the medial/epaxial and *Sim1* the lateral/hypaxial side of the boundary (Cheng et al., 2004). Moreover, *En1*-expressing cells originate from the epaxial dermomyotome precursor pool in the medial somite half, whereas *Sim1*-expressing cells originate from the lateral somite half (Olivera-Martinez et al., 2002; Cheng et al., 2004). *En1* is positively regulated by signals controlling the formation of epaxial somitic derivatives and repressed by signals controlling hypaxial development and *Sim1* expression (Olivera-Martinez et al., 2002; Cheng et al., 2004). Finally, in cell aggregation assays, *En1*- and *Sim1*-expressing cells sort out, suggesting that they may form a compartment boundary within the dermomyotome (Cheng et al., 2004). Eventually, *En1* expression spreads from the dermomyotome to the myotome and dermatome, maintaining a common expression boundary with *Sim1* (Cheng et al., 2004). However, the mechanism by which *En1* expression is installed in the myotome is not understood. Our data suggest that when mitotically active muscle precursors populate the myotome, they carry their dermomyotomal *En1* expression with them, thereby superimposing the molecular epaxial–hypaxial boundary from the dermomyotome onto muscle.

En1 Expression Sets a Molecular Boundary Within the Myotome

En1 expression in the dermomyotome and its association with the dermomyotomal epaxial–hypaxial compartment boundary is well documented (Cheng et al., 2004). However, the myotomal expression of *En1* and whether, when, and how this may relate to the epaxial–hypaxial segregation of muscle is not known. Analyzing the spatiotemporal expression pattern of *En1* and dermomyotomal, myotomal, and dermal marker genes, we found that, although *Alx4* expression was associated with a dermomyotomal–dermal fate of somitic cells and *Fgfr4*/*Pax3*/*Pax7*/*Paraxis* with a dermomyotomal-myotomal fate, *En1* expression was associated with both prospective dermal and muscle cells. However, among these two cell populations, *En1* only labeled a dorsomedially located subpopulation. From the initial dermomyotomal expression domain, *En1* expression spread into the underlying myotome and to the mesenchymal dermis above. Moreover, the *En1* expression boundaries in the dermomyotome, myotome, and dermatome were aligned. The *En1* expression domains in the dermomyotome, myotome, and dermatome laterally abutted the expression domain of *Sim1*. In the dermatome, *Sim1* expression spread into the *En1* domain at E4.5. However, this did not occur in the myotome. Thus, *En1* and *Sim1* expression domains are not associated with dermis/muscle formation per se, but rather molecularly define medial/epaxial–lateral/hypaxial subpopulations. Although

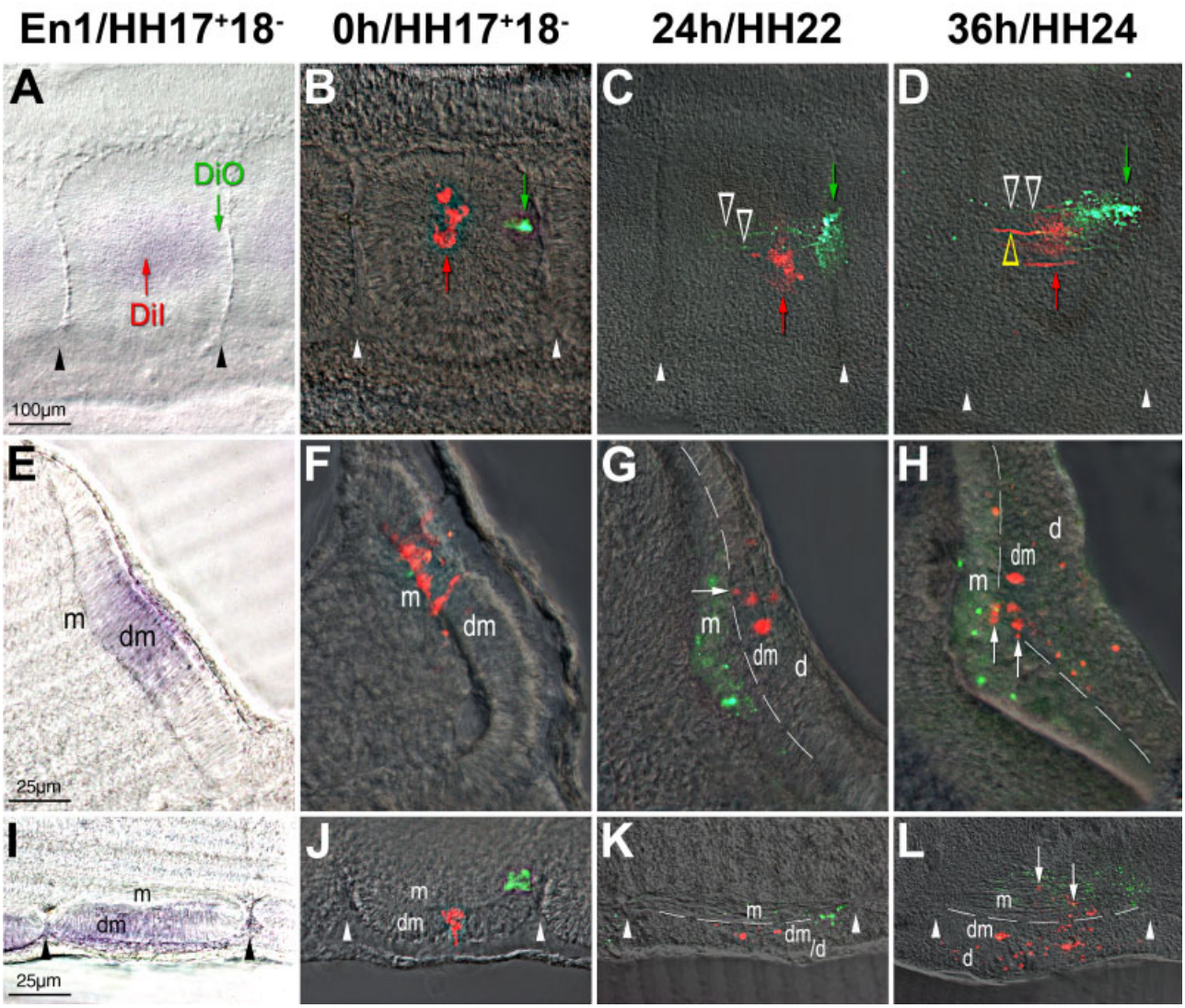

Fig. 5.

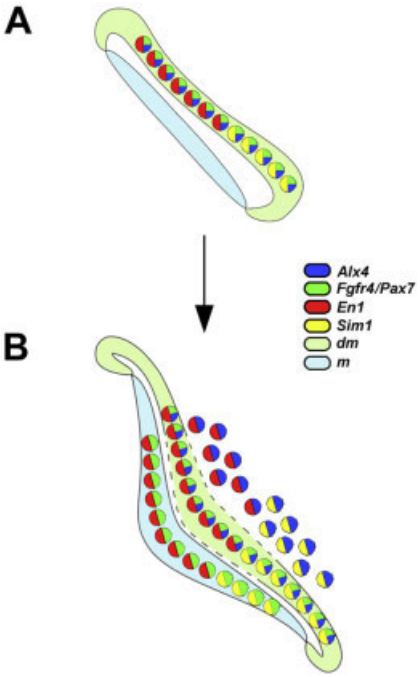

Fig. 6.

Fig. 5. Fate-mapping *En1*-expressing cells in different regions of the dermomyotome. **A,E,I:** Expression of *En1* in a stage IX flank somite at Hamburger and Hamilton stage (HH) 17^{+}. **B,F,J:** Labeling of a flank somite at this stage with DiI (center of the *En1*-expressing area) and DiO (rostral dermomyotomal lip of the *En1* domain). **C,G,K:** Flank somite at HH22, 24 hr after labeling. **D,H,L:** Flank somite at HH24, 36 hr after labeling. A–D: Dorsolateral views of whole somites, dorsal to the top, rostral to the right; sites of DiI and DiO labeling are indicated. E–H: The 50-µm Vibratome cross-sections; dml, top left corner; vll, bottom right corner. I–L: The 50-µm Vibratome frontal sections; medial to the top, lateral to the bottom, rostral to the right. Small arrowheads indicate the rostrocaudal borders of the somites, a broken white line indicates the dm/m interface. Abbreviations as in Figures 1 and 2. Scale bars = 100 µm in A (applies to A–D), 25 µm in E (applies to E–H), 25 µm in I (applies to I–L).

Fig. 6. Model for the superimposition of the epaxial–hypaxial boundary of the dermomyotome onto the myotome. The epithelially organized dermomyotome (dm) consists of dual-fated progenitor cells expressing *Alx4*$^{+}$/*Fgfr4*$^{+}$/*Pax7*$^{+}$. In the epaxial domain, these progenitor cells in addition express *En1*; in the hypaxial domain, they coexpress *Sim1*. Thus, the dm contains spatially segregated *En1*$^{+}$/*Alx4*$^{+}$/*Fgfr4*$^{+}$/*Pax7*$^{+}$ and *Sim1*$^{+}$*Alx4*$^{+}$/*Fgfr4*$^{+}$/*Pax7*$^{+}$ precursor cells. During de-epithelialization of the central dm, *Alx4*$^{+}$ cells fated for the dermatome move dorsally, maintaining the expression of *En1* and *Sim1*. Mitotically active *Fgfr4*$^{+}$/*Pax7*$^{+}$ muscle precursors move ventrally. They also maintain the expression of *En1* and *Sim1*. As a result, the molecularly defined epaxial–hypaxial boundary of the dermomyotome is superimposed onto the developing muscle and skin.

this molecular boundary in the dermatome may blur, *En1* and *Sim1* set up a molecular boundary in the myotome, which matches the original epaxial–hypaxial boundary in the dermomyotome.

En1 Expression Domain in the Dermomyotome Projects Onto the Myotome as Cells That Enter the Myotome Carry Their Dermomyotomal Expression Profile With Them

The myotome is laid down in waves (Christ et al., 1983; Denetclaw et al., 1997; Kahane et al., 1998a; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Huang and Christ, 2000; Cinnamon et al., 2001; Gros et al., 2004). The first muscle precursors are postmitotic and stem from the medial wall of the epithelial somite (Kahane et al., 1998b; Cinnamon et al., 1999). The next wave consists of postmitotic cells from the dermomyotomal lips (Kahane et al., 1998a; Cinnamon et al., 1999; Huang and Christ, 2000; Gros et al., 2004). Subsequently, mitotically active cells are generated from the rostrocaudal lips of the dermomyotome (third wave) and eventually, from the de-epithelializing dermomyotome proper (fourth wave; Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001; Ben-Yair and Kalcheim, 2005; Gros et al., 2005). Using fluorescent labeling, we found that cells within the *En1* expression domain contributed to the myotome first from the rostrocaudal lips, then from the de-epithelializing dermomyotomal center. Concomitant with the timing of muscle precursor production, *En1* expression spread from the dermomyotome into the myotome first from the rostrocaudal lips, then from the dermomyotome center; this spread of *En1* expression was continuous. Previous studies showed that there is little cell movement in the plane of the dermomyotome (Ordahl and Le Douarin, 1992; Kahane et al., 2001; Olivera-Martinez et al., 2002; Cheng et al., 2004). When dermomyotomal cells contribute to dermatome or myotome, they directly project into the territory above or beneath (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; this study). Taken together, this finding suggests that the *En1* expression domain projects directly into the myotome and dermatome as dermomyotomal cells carry their expression with them.

Molecular Subdivision of the Myotome Is Established Through Mitotically Active Muscle Precursors

The rostrocaudal lips first produce postmitotic muscle precursors (Christ et al., 1983; Denetclaw et al., 1997; Kahane et al., 1998b; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000). Subsequently, both the rostrocaudal lips and the dermomyotome proper generate mitotically active muscle precursors distinguished by the expression of *Fgfr4*/*Pax7*/*Paraxis* (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001). These cells respond to FGF molecules provided by the primary, postmitotic myotome and proliferate before withdrawing from cell cycle and forming elongated myocytes (Ben-Yair and Kalcheim, 2005; Gros et al., 2005). Previous studies established that *En1* expression commences in the dermomyotome of flank somites at stage VIII–X (Olivera-Martinez et al., 2002; Cheng et al., 2004). Our analysis indicates that soon thereafter, dermomyotome de-epithelialization and the formation of mitotically active muscle precursors is on its way. Significantly, *En1* expression reaches the myotome at the same time as the expression of *Fgfr4*/*Pax7*/*Paraxis*. Moreover, *En1* and *Fgfr4*/*Pax7*/*Paraxis* expression colocalizes, first at the rostrocaudal edges of dermomyotome spreading along the medial, sclerotome-facing surface of the myotome, then along the dorsal, dermomyotome-facing myotomal surface, spreading between the existing fibers. Thus, the appearance of *En1* expression in the myotome is concomitant with the appearance of mitotically active muscle precursors, first from the rostrocaudal dermomyotomal lips, then from the dermomyotomal center.

Fate mapping experiments indicated that epaxial–hypaxial cell lineages are established already at the time of gastrulation (Selleck and Stern, 1991; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002). However, somite rotation and heterotopic grafting of surrounding tissues showed that the epaxial–hypaxial fate is not yet determined and that epaxial–hypaxial fate of myogenic cells depends on extrinsic cues (Ordahl and Le Douarin, 1992). Moreover, the initial myotomal scaffold derived from first wave cells, at least at flank levels, is all-medial (Kahane et al., 1998b; Cinnamon et al., 1999). Even when the second wave myoblasts derived from the dermomyotomal lips populate the myotome (Kahane et al., 1998b; Cinnamon et al., 1999; Gros et al., 2004), no sign of any molecular subdivision except faint lateral *Sim1* expression is detectable. This finding suggests that the primary myotome consisting of postmitotic cells is not epaxially–hypaxially subdivided. Our data suggest that the first indication of the epaxial–hypaxial subdivision of the myotome is the onset of *En1* expression in this tissue, concomitant with the appearance of mitotically active muscle precursors. These integrate into the primary myotome, but through their mitotic activity, eventually outnumber the primary myotomal cells and constitute the bulk of the fetal and later newborn muscle (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Relaix et al., 2005). Thus, the epaxial–hypaxial subdivision of the myotome is established through mitotically active myoblasts.

En1 Expression May Serve as Positional Information for Satellite Cells

By tracing dermomyotomal cells to newborn and adult stages, it was shown that the dermomyotome proper delivers the muscles' satellite cells (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). These cells serve as an endogenous stem cell pool and repair muscle upon injury (Mauro, 1961; Charge and Rudnicki, 2004; Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Recent studies established that satellite cells have a recollection of the type of muscle they accompanied and, thus, are able to reconstitute muscle with the correct

combination of fast or slow-twitch fibers (Feldman and Stockdale, 1991; Donoghue et al., 1992; Dolenc et al., 1994; Kalhovde et al., 2005). Our study shows that *En1* marks the epaxial subset of muscle precursors originating from the dermomyotome proper, including the prospective satellite cells. Moreover, expression of *En1* as well as that of *Pax7* is maintained in these cells, whereas *Fgfr4*/*Paraxis*/*Pax3* expression declines. Ultimately, the *En1*-expressing part of the myotome will deliver the deep muscles of the back (Ordahl and Le Douarin, 1992; Denetclaw et al., 1997; Kalcheim et al., 1999; Denetclaw and Ordahl, 2000). These muscles are predominantly composed of slow-twitch fibers (Ng et al., 1998). It is possible, hence, that *En1* expression contributes to the specification of satellite cells as epaxial, predisposing them toward a particular regeneration program. Unfortunately, it is not known whether epaxial satellite cells, constitutively or upon activation, express *En1*. It is noteworthy, however, that the paralogous *En2* gene has been associated with fiber type selection in muscles of the jaw (Degenhardt and Sassoon, 2001).

Model for the Superimposition of the Epaxial–Hypaxial Boundary of the Dermomyotome Onto the Myotome

Our data suggest that the dermomyotome contains dual-fated progenitor cells, which generate mitosis competent dermal and muscle precursor cells. $Fgfr4^+/Pax7^+/En1^+$ and $Fgfr4^+/Pax7^+/Sim1^+$ cells in the dermomyotome immigrate into the myotome when the dermomyotomal center de-epithelializes. In a similar manner, *Alx4*-expressing cells in the dermomyotome maintain the expression of *En1* and *Sim1* as they move dorsally into the dermatome (Fig. 6). Thus, *En1* and *Sim1* are not associated with a particular cell fate but, rather, molecularly define epaxial–hypaxial subpopulations.

EXPERIMENTAL PROCEDURES

Chick Embryos

Fertilized hen (*Gallus gallus*) eggs were obtained from Winter Farm (Royston, UK) and Henry Stewart & Co., Ltd. (Lincolnshire, UK) and incubated at 38°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (1951), and the developmental age of flank somites was determined according to Christ and Ordahl (1995), modified by Pourquié (1999).

DiI/DiO Labeling

Fluorescent vital dyes DiI and DiO (Molecular probes) were diluted in ethanol to 0.5% DiI and 0.25% DiO. The dyes were injected into stage VII-I–XII flank somites of $HH17^+$–18^- (E2.75) embryos as described in Ruiz i Altaba et al. (1993). The labeling was performed under a Zeiss Stemi SV11 fluorescent stereomicroscope.

In Situ Hybridization

Whole-mount in situ hybridization was carried out as described in Dietrich et al. (1997, 1998) with avian-specific probes detailed in Mootoosamy and Dietrich (2002) for *Sim1*, *Pax3*, and *Myf5*; in Logan et al. (1992) for *En1*; in Šoˇsic et al. (1997) for *Paraxis*; in Goulding et al. (1994) for *Pax7*; in Takahashi et al. (1998) for *Alx4*; and in Marcelle et al. (1994) for *Fgfr4*.

Vibratome Sectioning

Embryos were embedded in 20% gelatin at 4°C and fixed in 4% paraformaldehyde for 4 days. Cross- and frontal sections of 50 μm thickness were obtained by using a Pelco 1000 Vibratome (Agar Scientific, UK).

Microtome Sectioning and Histology

Embryos for hematoxylin–eosin (H-E) staining were embedded in paraffin wax, and cross- and frontal sections of 12 μm thickness were obtained by using a Leica RM2245 Microtome (Leica Microsystems). A standard H-E protocol was followed.

ACKNOWLEDGMENTS

We thank Alisdair Edgar and Sharon Pudaruth for their help with the histological preparations. The study was supported by the Human Frontier Science Program, the European Network of Excellence Myores, and the Association Française contre les Myopathies.

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